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14. ABSTRACT

Background: iPS reprogramming to model 'disease-in-a-dish has become an attractive approach to study disease mechanisms. However, a frank pathophysiological phenotype in iPSC-derived PD neurons remains to be shown.
Objective/Hypothesis: The objective of this proposal is to accelerate the aging process of iPS-derived dopaminergic neurons with the goal of reproducing a Parkinson's disease (PD) specific phenotype in vitro.
Specific Aim 1.
To generate vector constructs and to establish Dox-inducible iPSC lines that express the Hutchinson-Gilford progeria (HGPS) gene (mutant lamin A with an in-frame loss of 150 nucleotides) and differentiate them into dopaminergic neurons.
Deliverable of this aim is the introduction of mutant lamin A in iPSC lines and optimization of inducible expression of mutant lamin A in iPSCs and during the differentiation into dopaminergic neurons.
Specific Aim 2.
To test whether iPSC-derived dopaminergic neurons transduced with mutant lamin A show changes in age-regulated genes at the mRNA and protein levels at different time-points during differentiation, thus exhibiting an accelerated aging.
Deliverable of this aim is the assessment of specific age-related aging pattern of gene and protein expression in induced mutant lamin A modified iPSC-derived neurons.
Specific Aim 3.
To test whether induced mutant lamin A cell lines differentiated into dopaminergic neurons exhibit hallmark pathology of PD, such as protein aggregation of alpha-synuclein, posttranslational modification, and signs of mitochondrial pathology.
Deliverable of this aim is the assessment of the pathological PD-related phenotype in induced mutant lamin A modified iPSC-derived neurons.
Study Design: This is an in vitro study of patient-specific iPSC-derived dopaminergic neurons in which truncated lamin A will be introduced to study cellular phenotypes under the hypothesis that cells under expression of lamin A will age faster than untreated cells.
Relevance: Creation of iPSC lines from patients with PD that develop a disease phenotype would revolutionize research in PD and could produce more predictive disease models to enable the advancement of better candidates into clinical testing. If successful, the impact of this research project on the iPSC field could be enormous. It could remove one of the remaining roadblocks to using iPSC model for PD for the study of disease mechanism and drug development, which could bring us closer to finding the cause and cure for PD.

15. SUBJECT TERMS
Parkinson's disease, induced pluripotent stem cells, cellular model, accelerated aging, lamin A, progerin

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A. Introduction:

The overall goal of the project is to accelerate the aging process of neurons derived from induced pluripotent stem cells donated by Parkinson's disease patients. The overall goal is to introduce mutated lamin A (progerin) to accelerate the aging process and to evaluate phenotypic differences related to Parkinson's disease. Proof-of-concept studies with lentiviral constructs have been recently published showing that our approach is valid and can be successful (Miller et al. 2013).

B. Body:

1. Establishing a double stable RetroX Tet-on advanced neural stem cell lines from a SNCA triplication carrier, MSA case, and normal control and characterize lines for both elements of rtTA and GFP-Progerin

We tested the efficacy of plasmids of pRetroX Tet-on and pRetroX-Tight-Pur-GFP-Progerin and test whether Tet-on and GFP-progerin will be expressed in the Huf5 NSC cell lines after co-transfection of those plasmids into NSCs and later on adding Doxycycline to induce expression. Transfection and following Western blot experiment are on-going (Figure 1).

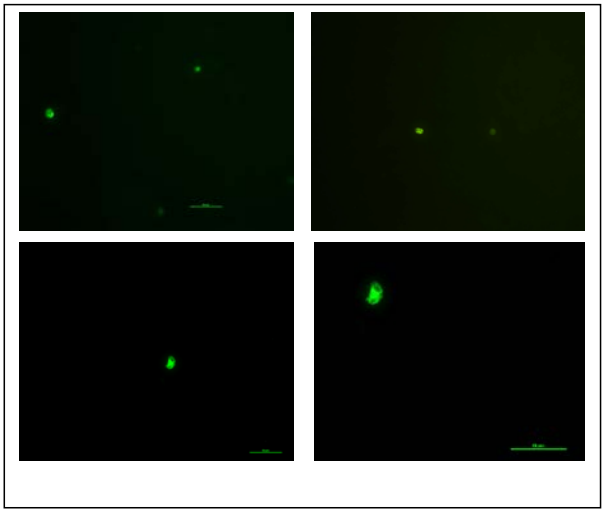

Figure 1. Transfection of pRetroX-Tight-Pur-GFP-Progerin in neural stem cells. Green cells show the expression of green fluorescence protein (GFP).

Experimental approach: Briefly, $2.5*10^5$ NSCs were plated on one well of 24-well plate. The next day, when the cells reached 80% confluency, we started transfections. 500ng of pRetroX-GFP-progerin and 500 ng of pRetro-Xtet-on plasmid were added to 50 ul of OptiMEM and incubated at room temperature for 5 min. At the same time, 1.0 ul of Lipofectmine was added to another 50 ul of OptiMEM and incubated at room temperature for 5 min. Both solutions were mixed together and incubated for another 20 min and added to the cells. The next day after transfection, we changed the medium containing 500 ng/ml Doxycycline, and we observed the GFP-Progerin expression in the NSC the next day after adding doxycycline (Figure 1).

After testing, we will make both serial infection and co-transduction of pRetroX-Tet-on and pRetroX-Tight-Pur-GFP-Progerin to establish HUF4, HUF5 and MSC PI-1857 double stable RetroX-Tet-On advanced neural stem cell lines, expressing both rtTA and GFP-Progerin proteins. Antibiotics G418 and puromycin will be used to select the double stable cell lines. GFP should help with selecting positive clones. Western blot analysis will be used to detect the protein expression after selection.

2. Differentiation into oligoprecursor cells (OPCs) and oligodendrocytes

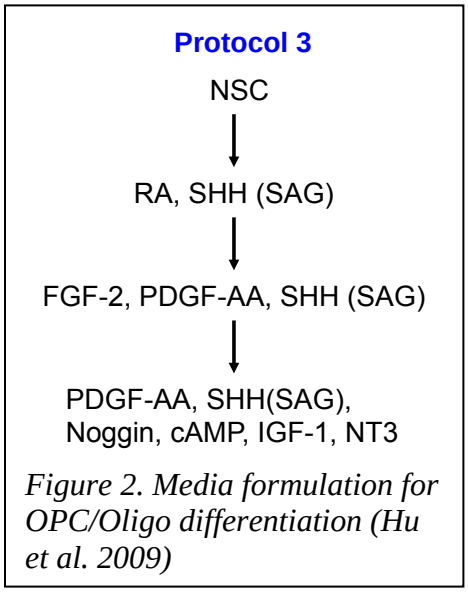

Figure 2. Media formulation for OPC/Oligo differentiation (Hu et al. 2009)

In the last report, we discussed the initial optimization experiments to generate OPCs. We made progress deriving oligoprecursor cells from neural stem cells and are now in the process of differentiating them into oligodendrocytes (Figure 2 and Figure 3).

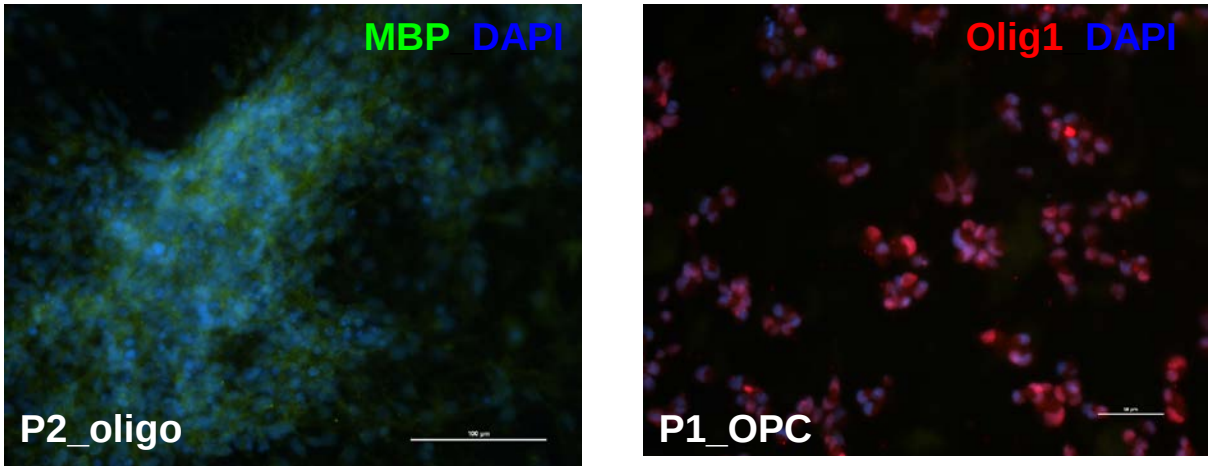

Figure 3. Immunohistochemistry for myelin binding protein (MBP) in green and oligodendrocyte transcription factor 1 (Olig1) in red. DAPI in blue is the nuclear counterstain.

3. Comparing aged versus control neurons for signs of cellular aging

One aspect of the project is to characterize aging *in vitro*. We compared different passage numbers of neural stem cells (NSCs) and their potential to differentiate into neurons. We

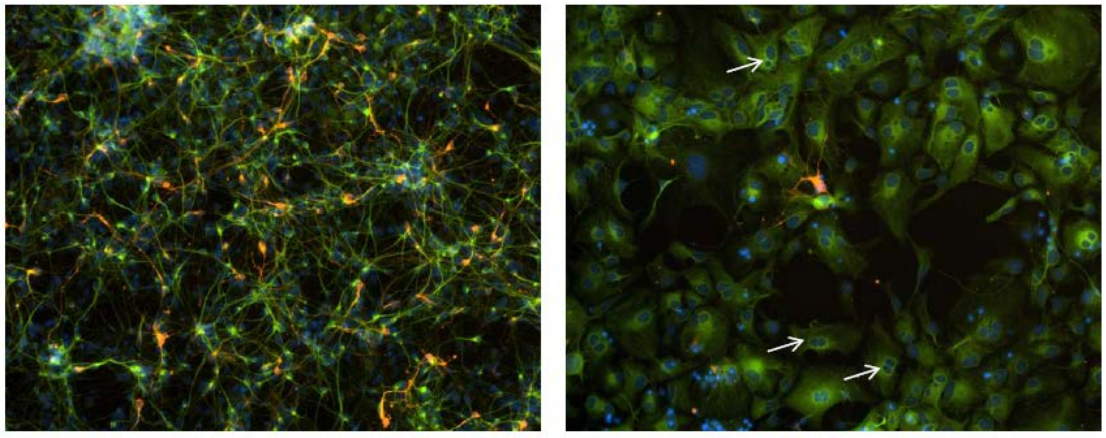

Figure 4. Differentiated neurons stained for neuronal marker MAP2 (green) and dopamine marker (TH, orange); left: neurons derived from NSCs at passage 10, right: cultures from NSCs at passage 34. Arrows indicate multinucleated cells.

used NSCs from passage 10 and passage 35 and noted characteristic signs of cellular aging like increased cell size, nuclear size, increased number of multinucleated cells, increased cytoplasmic microfilament (Figure 4). We anticipate observing these changes in the progerin modified lines at earlier passages.

4. Additional age-related markers for the characterization of Progerin lines

In order to identify the aging marker, we will compare various aging markers and neural markers in DA neurons and oligodendrocytes. Besides the morphological changes, we will assess surface marker expression of lamin B1, LAP2, y-H2AX, and 53BP1, We will also use a senescent cell staining kit (Sigman Aldrich) to stain from SA-b-galactose.

The gene array assay will be prepared by Life Technologies Company as the Taqman gene expression panel: IGF-1, EGF, c-fos, MMP1, IL6, CyclinD1, ELASTIN, Collagen Type 1, Retinoblastoma, GADD45, TERT, P53, P21, Zmpste24. This characterization will allow us to assess the cellular aging process of the cells that we have introduced by introduction of progerin.

5. Establishing a double stable RetroX Tet-on advanced neural stem cell lines

We are performing a co-transfection with pRetroX-Tet-on and pRetroX-Tight-Pur-GFP-Progerin to establish double-stable NSC lines expressing the rtTA and GFP-Progerin proteins. We are deriving lines now from a Parkinson's disease patient with a triplication in the alpha synuclein gene, a patient with a severe form of parkinsonism, multiple system atrophy, and matched controls.

6. Differentiation into dopaminergic neurons and oligodendrocytes

In the last progress report, we reported on the functional analysis of differentiated neurons for dopamine release and electrical spiking pattern.

Now, we are establishing the differentiation of glial cells to ultimately being able to model completely functional circuits very similar to in vivo conditions. We have derived oligoprecursor cells from neural stem cells and in the process of differentiating them into oligodendrocytes. Alpha-synuclein accumulation has been reported in oligodendrocytes in the brain of patients with certain forms of parkinsonism.

7. Project plan for the 9 month extension period:

Technical Objective 4: differentiate engineered iPSCs into DA neurons and oligodendrocytes

Technical Objective 5: evaluation of these differentiated neurons for an age-related phenotype by measuring regulation of key genes associated with aging and markers the different neuronal and glial lineages

Technical Objective 6: Evaluation of these differentiated neurons for a pathological phenotype related to PD by assessing protein aggregation of alpha-synuclein, posttranslational modification, and signs of mitochondrial pathology.

8. High-performance liquid chromatography for dopamine release in differentiated iPSC neurons

In order to deem the iPS neuron model successful, we need to functionally validate the cultures. In this reporting period, we are reporting on the functional characterization of dopaminergic neurons for dopamine release and detection of spike pattern using multielectrode arrays.

After 60 days of neuronal differentiation, media was removed and 1 ml of Neurobasal/N2 media supplemented with 56mM KCl was added per well (6-well dish) and incubated at 37C for 15 min. The K+ stimulation induces dopamine release from the neurons.

Media was then collected and immediately frozen in liquid nitrogen and stored at -80C until further use.

Subsequently, protein lysates of each well were made in ice cold 25 mM Tris supplemented with a Complete Mini protease inhibitor cocktail tablet (Roche). Lysates were sonicated and cleared by maximum speed centrifugation in a tabletop microcentrifuge. Soluble protein concentrations were measured by a standard Bradford assay. KCl stimulated samples were thawed, stabilized at a final concentration of 0.4N perchloric acid, and centrifuged at 15,000 rpm at 4C for 12 min to clear debris. Supernatant was collected and dopamine assayed by HPLC with electrochemical detection (Coularray detector, ESA, Chelmsford, MA) using a reverse phase C18 column (Perkin Elmer Instruments, Shelton, CT). The mobile phase consisted of a mixture of 90 mM sodium acetate, 35 mM citric acid, 130 uM ethylene-diamine-tetra-acetic-acid (EDTA), 230 uM 1-octanesulfonic acid and 10% (v/v) methanol, with a flow rate of 1mL/min. DA concentration was quantified by comparison of AUC to known standard dilutions (0.3ng/ml-30ng/ml). Well to well variation was adjusted by protein determinations of cleared protein lysates, see Figure 1.

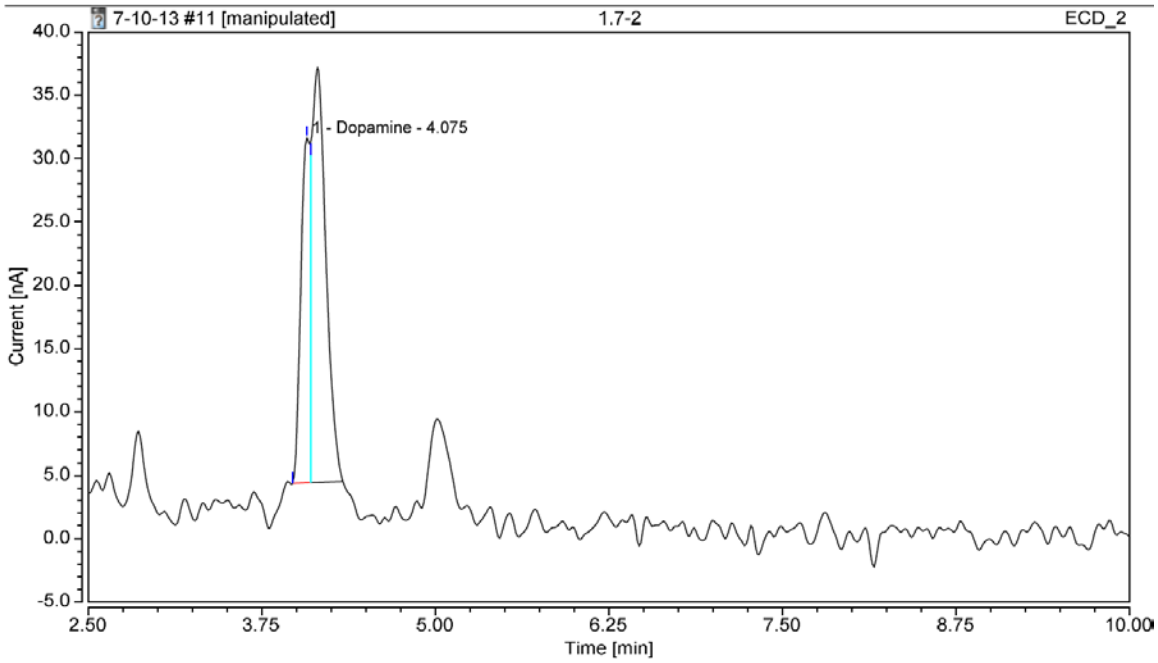

Figure 1. Dopamine measurement shows peak with retention time 4.075 min which relates to 3.5ng/ml dopamine.

This is the proof that these cultures indeed produce dopamine and can potentially be functionally active. In the next step, we used multielectrode arrays to test for electrical activity.

9.

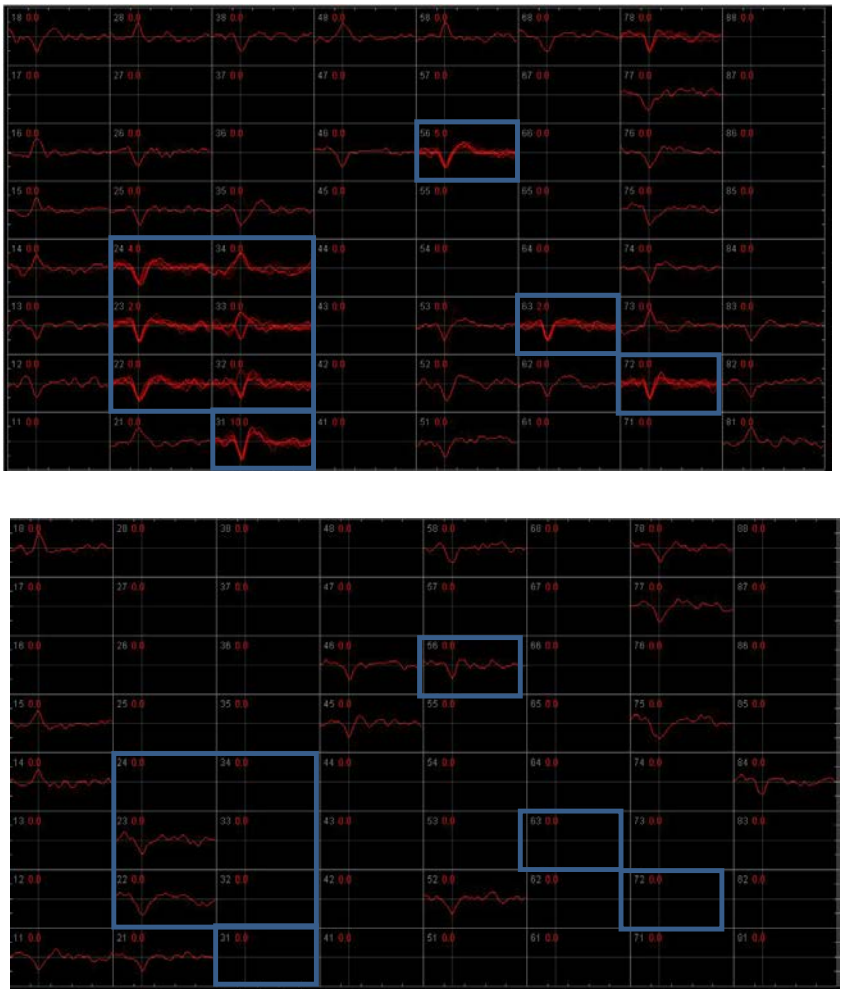

Figure 2. A: 35 day iPS neuronal culture network recording before stimulation, accumulated reading after 8 min recording time; B: same culture after treatment with 1uM tetrodotoxin shows abolishment of spikes.

Microelectrode Arrays for measurement of electrical activity in iPS derived neurons

To proof that the neurons in the culture dish are electrically active and functional, we decided to use the technology for microelectrode arrays (MEAs) which allows for extracellular recordings of neuronal activity. The rational for using MEA technology of single cell patch-clamping is the ease of use of the system and that the cultures can be continuously measured over time with no destructive interference to the tissue being investigated.

The MEAs are made up of a grid of 64 electrodes, and each electrode is capable of simultaneously monitoring the activity of individual cells. The arrangement of multiple electrodes provide concurrent access to both single cell or network-level activity.

In Figure 2, we measured 35 day old iPS derived neurons that were growing on an MEA electrode plate. We measured a spontaneous base spike activity at the electrodes boxed in blue. After stimulation with 1uM tetrodoxin, the spike activity was abolished with on single, one time spikes recorded in three of the ten previously active electrodes.

We believe that these assays are critical to validate the iPS derived neurons as an in vitro model. We can now further age these neurons to in more depth assess the changes during the aging process in the culture dish to examine the PD and age-related phenotypes.

10. Co-transfection of iPS lines with Tet-on and progerin:

We were performing for this reporting period a co-transfection with pRetroX-Tet-on and pRetroX-Tight-Pur-GFP-Progerin to establish double-stable iPS lines expressing the rtTA and GFP-Progerin proteins. *These lines are pending and are being characterized.*

11. Advancement of Neuronal differentiation

After generation of neural stem cell, reported in last reporting period, we are now differentiating the neural stem cells into mature dopaminergic neurons. This protocol takes 35 days and requires two different media, first neurobasal media supplemented with FGF8 and smoothened agonist (SAG) for 10 days, then a combination of BDNF and GDNF in neurobasal media for 25 days. Markers for TH, beta-tubulin, MAP2, lamin A, and various transcription factors such as Lmx1A, Otx2, and FoxA2 will be assessed for midbrain specificity. *Neuronal differentiation is underway, cultures are around day 20.*

12. Establishment of aging markers

One goal of the proposal as part of Milestone 5, we want to define an age-related phenotype. One established marker is the expression of the tau isoform 3R and 4R (Iovino et al., 2010). We are currently testing the expression in naïve or untransfected stem cell cultures in the alpha-synuclein triplication cell lines compared to the control

lines and will have the assay optimized once the progerin lines are derived. In stem cell lines we are not expecting any expression of tau, however, at the NSC stage, we expect mostly the 3R form of tau to be expressed, whereas in the differentiated neurons we would expect a ratio of 1:1 for 3R versus 4R isoform. These experiments are underway. RNA has been collected and PCR optimization is pending.

Other age-related changes that have been reported to be modified in human are also tested (Loerch et al., 2008). We will determine expression of differentially regulated genes as described in Table 1.

Human_Gene	Mouse_Gene	Rhesus_ Accession	Human_Fold	Gene
D4S234E	Nsg1	XM_001095887	-1.610	Dopamine
GABRB3	Gabrb3	XM_001109060	-2.850	GABA
SLC32A1	Slc32a1	XM_001089139	-2.440	GABA
GABARAPL1	Gabarapl1	XM_001115102	-2.250	GABA
GABRA1	Gabra1	XM_001086287	-2.16	GABA
GABRG2	Gabrg2	XM_001087472	-2.160	GABA
GAD1	Gad1	XM_001083231	-2.020	GABA
GABRA5	Gabra5	XM_001109231	-1.950	GABA
GAD2	Gad2	XM_001101800	-1.560	GABA
DDX1	Ddx1	XM_001090643	-2.180	GABA
GABRB2	Gabrb2	XM_001085738	-1.66	GABA
HOMER1	Homer1	XM_001109170	-1.890	Glutamate
GRIK1	Grik1	XM_001100491	-1.7	Glutamate
SLC1A1	Slc1a1	XM_001085339	-1.62	Glutamate
ARL6IP5	Arl6ip5	XM_001087809	-1.540	Glutamate
CDK5R1	Cdk5r1	XM_001113136	-1.520	Glutamate
GRIA1	Gria1	XM_001111339	-1.81	Glutamate
FOLH1	Folh1	XM_001096141	1.670	Glutamate
GLRB	Glrb	XM_001093832	-1.990	Glycine
IQWD1	Iqwd1	XM_001091473	-1.620	Glycine
OPA1	Opa1	XM_001087037	-1.54	Glycine
CALB1	Calb1	XM_001085269	-3.750	Calbindin
SST	Sst	XM_001103516	-3.070	somatostatin
PNOC	Pnoc	XM_001110309	-2.160	Prepronociceptin
VIP	Vip	XM_001096218	-2.110	Vasoactive intestinal peptide
TAC1	Tac1	XM_001089754	-2.080	Tachykinin
CCKBR	Cckbr	XM_001102094	-1.700	Cholecystonchinin B receptor

Table 1. List of genes that are differentially regulated in human, rhesus monkey and mouse which will be tested in iPSC derived neurons aged with progerin.

Key Research Accomplishments:

- *Milestone 1* (month 1-2): generation of the drug-inducible construct using Clontech Retro-X Tet-On advanced vector **COMPLETED**
- *Milestone 2* (month 2-3): establishing stable iPSC clones with drug-inducible progerin construct **we established the plasmid transfection for iPSC induced neural stem cells, the retroviral selection is in PROGRESS**
- *Milestone 3* (month 4-6): differentiation into DA neurons and oligodendrocytes for all generated lines **COMPLETED for Neurons, OPTIMIZATION PHASE for Oligodendrocytes**
- *Milestones 4* (month 6-12): examination of DA-phenotype, age-related phenotype, and pathological phenotype related to PD **OPTIMIZATION PHASE**

Reportable Outcomes:

At this point, no publications or posters have been derived from the grant.

Conclusions:

Creation of iPSC lines from patients with PD that develop a disease phenotype would revolutionize research in PD and could produce more predictive disease models to enable the advancement of better candidates into clinical testing.

References:

Iovino, M., Patani, R., Watts, C., Chandran, S., and Spillantini, M.G. (2010). Human stem cell-derived neurons: a system to study human tau function and dysfunction. PloS one *5*, e13947.

Loerch, P.M., Lu, T., Dakin, K.A., Vann, J.M., Isaacs, A., Geula, C., Wang, J., Pan, Y., Gabuzda, D.H., Li, C.*, et al.* (2008). Evolution of the aging brain transcriptome and synaptic regulation. PloS one *3*, e3329.

Miller, J.D., Ganat, Y.M., Kishinevsky, S., Bowman, R.L., Liu, B., Tu, E.Y., Mandal, P.K., Vera, E., Shim, J.W., Kriks, S., *et al.* (2013). Human iPSC-Based Modeling of Late-Onset Disease via Progerin-Induced Aging. Cell stem cell *13*, 691-705.

Hu BY, Du ZW, Zhang SC: Differentiation of human oligodendrocytes from pluripotent stem cells, Nat Protoc 2009, 4:1614-1622

Sharla M. O. Phipps, Joel B. Berletch, Lucy G. Andrews, and Trygve O. Tollefsbol: Aging Cell Culture: Methods and Observations, Methods Mol Biol. 2007 ; 371: 9–19

Appendices:

None